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Comparison of test systems for RNA*interference*

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Abstract

RNA*interference* (RNAi) has developed within a short time from an area of basic research occupied by a few experts to a widely used technical tool for reverse genetics, which is expected to have a broad utility not only in research, but also in medical and diagnostic applications. Despite its widespread use, the application of RNAi is often hampered because a difference of only a few nucleotides in the sequence of the target RNA can change the efficiency of a small interfering RNA (siRNA) from high to zero, and publicly available design tools for siRNAs are not yet perfect. We therefore developed and compared RNAi test systems based on different promoters, reporters, and target sequences. Here, we show that fluorescence-based test systems have obvious disadvantages compared to luciferase-based test systems and that some combinations of promoter, reporter, and target sequences, although currently in use, are not well suited for testing RNAi effects.

Keywords: RNA*interference*; Test systems; Fluorescence; Luciferase; Promoters

RNA*interference* or RNAi is the inhibition of gene expression by double-stranded RNA [1]. Recently, RNAi by small interfering RNAs (siRNAs) or short hairpin RNA (shRNA) has become a widely used tool for artificial manipulation of gene expression in cells of higher eukaryotes, which is usually achieved by transfecting cells with either siRNAs or shRNA-expressing vectors. SiRNAs are synthetic double-stranded RNA molecules that correspond to a target sequence of an expressed mRNA. They are 19–23 base pairs in length and have two-nucleotide 3′ overhangs. ShRNAs are synthetic or naturally occurring single-stranded transcripts with a short hairpin (sh) structure and a length of approximately 64 nucleotides; the hairpin also consists of a target sequence in sense and antisense orientation that is separated by a spacer group [2].

One obstacle in the application of RNAi is the large difference in the efficiency of siRNAs to down-regulate the expression of a given target sequence. Even a slight shift of only a few base pairs in the target position can change an efficient siRNA to an siRNA with no or only minor effects [3,4]. The sequence of an siRNA also determines whether an siRNA provokes an interferon response [5,6] or other off-target effects in a given cell type [7–10], although sequence-independent activation of the interferon response by siRNAs has also been reported [11].

Recently, several laboratories have developed algorithms based on sequence-specific and thermodynamic parameters in order to design efficient and specific siRNAs [12–17]. But predictions made with the help of different publicly available design tools are not consistent and can differ considerably. Furthermore, possible off-target effects can only be calculated if sufficient sequence data for species-specific BLAST searches are available. As a result, in most cases, researchers are left with the task of screening multiple siRNAs in order to identify a suitable one. This is especially cumbersome when the development of a knock-down phenotype is difficult to achieve or time-consuming [18]. Hence, it would be of great advantage to check the efficiency of a designed siRNA before embarking on labour-intense knock-down experiments such as, for

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example, the in vivo application of viral RNAi vectors. We therefore developed and compared different test systems for RNAi. All test systems consisted of a promoter directing the expression of a transcript that encoded a reporter sequence (such as EGFP or luciferase) with a stop codon, which was fused to a target sequence. This study shows that the right combination of promoter, reporter, and target sequences is critical for a successful evaluation of RNAi function and that some combinations are not suited to investigate RNAi effects.

Materials and methods

Vector construction. pSUPER- and pAAV-vectors containing expression cassettes for shRNAs and the red-fluorescent protein DsRed have recently been described [21]. pSUPER, pGEM4Z, and pGL3 vectors containing the different expression cassettes for the reporter and target sequences were cloned according to standard methods. Cloning products were sequenced to confirm their identity. A schematic outline of the test vectors and shRNA-expressing vectors is shown in Fig. 1 and Table 1 shows the names and features of the vectors used in this study.

The sense sequence of the EGFP shRNA was AAGCTGACCCTGAA GTTCA, the sense sequence of the luciferase shRNA1 was GTGCGCT GCTGGTGCCAAC [20], the sense sequence of luciferase shRNA2 was GTGCGCTGCTGGTGCCAACCC [19], the sense sequence of the RhoA-specific shRNA1 was GACATGCTTGCTCATAGTCTT [22], the sense sequence of the RhoA-specific shRNA2 was CAGATATTGAA GTGGACGGGA, and the sense sequence of the RhoA-specific shRNA3 was CTATGTGGCAGATATTGAAGT. The following target sequences were used: For EGFP the complete ORF of 720 bp was cloned behind the stop codon of the respective reporter sequence. For the long luciferase target sequence a fragment of 472 bp (base pair 751–1222) was amplified from the pGL3-promoter vector from Promega (Mannheim, Germany), and the short luciferase target sequence was a fragment of 121 bp encompassing base pair 915–1035 of the pGL3-promoter vector. The RhoA target sequences were amplified from rat brain cDNA and had a length of either 582 bp (corresponding to the complete ORF) for the long RhoA target sequence (base pairs 178–759 form Accession No. BC061732) or a length of 164 bp for the short RhoA (corresponding to base pairs 199–362 of the cDNA sequence). All target sequences were cloned as 3′-untranslated regions of the reporter sequence.

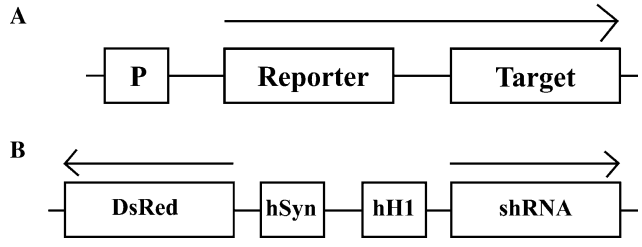

Fig. 1. Schematic outline of the test vectors (A) and shRNA-expressing vectors (B). The test vectors (A) contained one of the following four different promoters (P): mouse cytomegalovirus promoter (CMV), simian virus40 promoter (SV40), human U1 promoter (hU1), or human synapsin gene1 promoter (hSyn). As a reporter (Reporter) one of the following four different proteins was used: EGFP, dEGFP, DsRed2N1, or luciferase firefly. As target sequences (Target), either long (>470 bp) or short (<170 bp) sequences were used. (B) The shRNA-expressing vectors. For a more detailed description of the different vectors used in this study, please also refer to Table 1. The arrows above the drawings represent the direction of transcription. Please note that the outline is not drawn to scale.

Cell culture. HEK 293 cells (AAV-293 cells) were from Stratagene (Heidelberg, Germany). Cells were passaged in 175 cm^2 bottles at 50–60% confluency, and for this study, only passages 10–24 were used. Cells were seeded at a density of 7×10^5 cells per well in six-well plates, and 24 h after seeding, the cells were transfected with plasmids by the calcium phosphate method according to standard protocols [27]. Transfection was performed with 30 μg plasmids per well and a molar ratio of reporter- and shRNA-expressing plasmids of 1:4. Transfection rates varied between 30% and 80% and were usually above 50%.

Microscopy, luciferase assay, INF-α assay, and statistical analysis. Pictures of live cultures were taken with an Axioplan 2 microscope from Zeiss (Germany), and further analysis was done with the Axio Vision 3 software. The luciferase assay was from BD Pharmingen (NJ, USA) and was performed according to the instructions of the manual, except that 5 μl of sample and 50 μl of solutions A and B were used. Luminescence was measured with a liquid scintillation and luminescence counter from Wallac (Freiburg, Germany), and data were evaluated with the MicroBeta Windows Workstation program. Samples collected from cells transfected with CMV-luciferase reporter constructs were diluted 1:10 in lysis buffer prior to analysis. Interferon alpha was measured using a human INF-α ELISA kit from PBL Biomedical Laboratories (NJ, USA) with the extended range protocol. GraphPad Prism 3.0 Software (GraphPad, San Diego, California, USA) was used to perform statistical analyses and graphical presentation. All data are expressed as means ± SD. Each group analysed consisted of three independent samples, and all experiments were repeated at least twice.

Results

For transfection we used HEK 293 cells because they can easily be transfected with plasmids by the calcium phosphate method. Although other investigators detected a slight interferon response with their HEK cell line after treatment with high concentrations of chemically or enzymatically synthesised siRNAs [11], we did not observe any INF-α production with our cell line after challenging with up to 8 μg (approximately 1.5 pmol) shRNA-expressing plasmid per well, or stimulation with 100 U INF-γ together with 1 μg/ml lipopolysaccaride, or with 100 μg/ml polyI:C. The lack of INF-α production by HEK cells excluded off-target effects caused by an interferon response.

The first RNAi test vector constructed by us consisted of a pGEM4Z vector backbone that contained a mouse cytomegalovirus promoter (CMV) directing the expression of a transcript encoding EGFP (reporter) fused to 472 bp of the coding sequence from firefly luciferase (pGEM-CMV-EGFP-long-luc-test). The shRNA vectors expressed DsRed from the human synapsin gene1 promoter (hSyn) and one of two different luciferase-specific shRNAs [19,20] from the human H1-promoter (hH1) (pAAV-hSyn-DsRed-shRNA-luc1 and -luc2). As positive control a recently described vector expressing an shRNA directed against the reporter protein EGFP was used (pAAV-hSyn-DsRed-shRNA-EGFP) [21]. When HEK cells were transfected with pGEM-CMV-EGFP-long-luc-test (Table 1) and with one of the luciferase shRNA-expressing vectors, we did not see any significant changes in the expression of EGFP as compared to controls without luciferase shRNA expression. When cells were transfected with pGEM-CMV-EGFP-long-luc-test and the EGFP shRNA-expressing vector, the green fluorescence was

Table 1
Features of the test vectors and shRNA-expressing vectors

Name	First promoter	First transcript	Second promoter	Second transcript
pGEM-CMV-EGFP-long-luc-test	mCMV	EGFP-long-luciferase		
pAAV-hSyn-DsRed-shRNA-luc1	hSyn	DsRed	hH1	luciferase shRNA1
pAAV-hSyn-DsRed-shRNA-luc2	hSyn	DsRed	hH1	Luciferase shRNA2
pAAV-hSyn-DsRed-shRNA-EGFP	hSyn	DsRed	hH1	EGFP shRNA
pGEM-CMV-DsRed-EGFP-test	CMV	DsRed-EGFP-long-luciferase		
pGEM-CMV-dEGFP-long-luc-test	mCMV	dEGFP-long-luciferase		
pAAV-hSyn-DsRed-CytbAS	hSyn	DsRed	Disabled hH1	None
pGEM-hSyn-EGFP-long-luc-test	hSyn	EGFP-long-luciferase		
pMH4-U1-EGFP-long-luc-test	hU1	EGFP-long-luciferase		
pGEM-U1-EGFP-short-luc-test	hU1	EGFP-short-luciferase		
pGEM-hSyn-EGFP-short-luc-test	hSyn	EGFP-short-luciferase		
pMH4-SV40-short-luc-test	SV40	EGFP-short luciferase		
pGEM-U1-dEGFP-short-luc-test1	hU1	dEGFP-short-luciferase		
pGEM-U1-short-RhoA-test1	hU1	EGFP-short-RhoA		
pAAV-hSyn-DsRed-shRNA-RhoA1	hSyn	DsRed	hH1	shRNA-RhoA1
pAAV-hSyn-DsRed-shRNA-RhoA2	hSyn	DsRed	hH1	shRNA-RhoA2
pAAV-hSyn-DsRed-shRNA-RhoA3	hSyn	DsRed	hH1	shRNA-RhoA3
pGL3-SV40-luc-EGFP-test	SV40	Luciferase-EGFP		
pGL3-U1-luc-EGFP-test	hU1	Luciferase-EGFP		
pGL3-CMV-luc-EGFP-test	mCMV	Luciferase-EGFP		
pGL3-SV40-luc-long-RhoA-test	SV40	Luciferase-full-length-RhoA		
pGL3-SV40-luc-short-RhoA-test1	SV40	Luciferase-short-RhoA		
pGL3-SV40-luc-short-RhoA-test2	SV40	Luciferase-short-RhoA-reverse		

Names and characteristics of the plasmids used in this study. The first column gives the names of the plasmids; the second column lists the type of the polymerase II promoter directing the expression of the test system or the fluorescent protein only, respectively. The third column gives the names of the transcripts from the polymerase II promoter expression cassette. Column four lists the polymerase III promoter directing the expression of the shRNA, and column five lists the names of the shRNAs expressed from the polymerase III expression cassette.

slightly reduced. When EGFP was substituted by DsRed as the reporter and the luciferase target sequence by the complete coding sequence of EGFP fused to the luciferase sequence (pGEM-CMV-DsRed-EGFP-test), again, no reproducible down-regulation of DsRed by the EGPF shRNA was observed (data not shown). We reasoned that these results were likely due to the combination of a very strong promoter expressing proteins with long half-life periods (such as EGFP and DsRed). Therefore, we changed EGFP for the destabilised form of EGFP (dEGFP; half-life period of 2 h) in the pGEM-CMV-EGFP-long-luc-test vector. But again, cells transfected with the dEGFP-luciferase-expressing vector (pGEM-CMV-dEGFP-long-luc-test) and with one of the luciferase shRNA-expressing vectors did not show any substantial down-regulation of the EGFP fluorescence as compared to negative controls without shRNA. In contrast, cotransfection of HEK cells with pGEM-CMV-dEGFP-long-luc-test and the EGFP shRNA-expressing vector resulted in an obviously decreased EGFP fluorescence as compared to cells transfected with pGEM-CMV-dEGFP-long-luc-test only (Fig. 2).

Next, we examined which promoters other than the CMV promoter were able to direct the expression of EGFP in HEK 293 cells. Besides the human synapsin gene1 promoter, the human U1 promoter and the simian virus40 promoter (SV40) directed the expression of EGFP in HEK cells. However, addition of the long luciferase target sequence to the sequence of EGFP resulted in a distinct decrease in the fluorescence expressed from U1 or from the human synapsin promoter, and even shortening of the target sequence from 472 to 121 bp only partially solved the problem (Fig. 3). This very obvious reduction in the expression level of green fluorescence caused by the addition of the target sequences to the EGFP reporter sequence is equivalent to a large decrease in the dynamic range available for regulation of an EGFP fusion transcript by shRNAs. Therefore, in the case of the SV40 promoter, only the construct with the short target sequence was cloned. A comparison of the green fluorescence of cells transfected with pGEM-U1-EGFP-short-luc-test and cells transfected with pMH4-SV40-EGFP-short-luc-test revealed a less intense green fluorescence in cells transfected with the SV40 construct as compared to the fluorescence seen in cells transfected with the human U1 promoter construct (data not shown).

We then analysed the effect of various shRNAs on the level of U1 promoter-directed EGFP fluorescence in HEK cells. As shown in Fig. 4, a U1 promoter-directed EGFP-short luciferase transcript can be down-regulated by a luciferase shRNA, although in experiments with low transfection rates, the results were sometimes difficult to interpret (Fig. 4A). When dEGFP was used as the reporter, the general fluorescence was usually less than the fluorescence seen with EGFP. Although down-regulation of the EGFP fluorescence by luciferase shRNA was visible at earlier time points with the dEGFP, we did not find obvious differences in the general regulation of the two reporters.

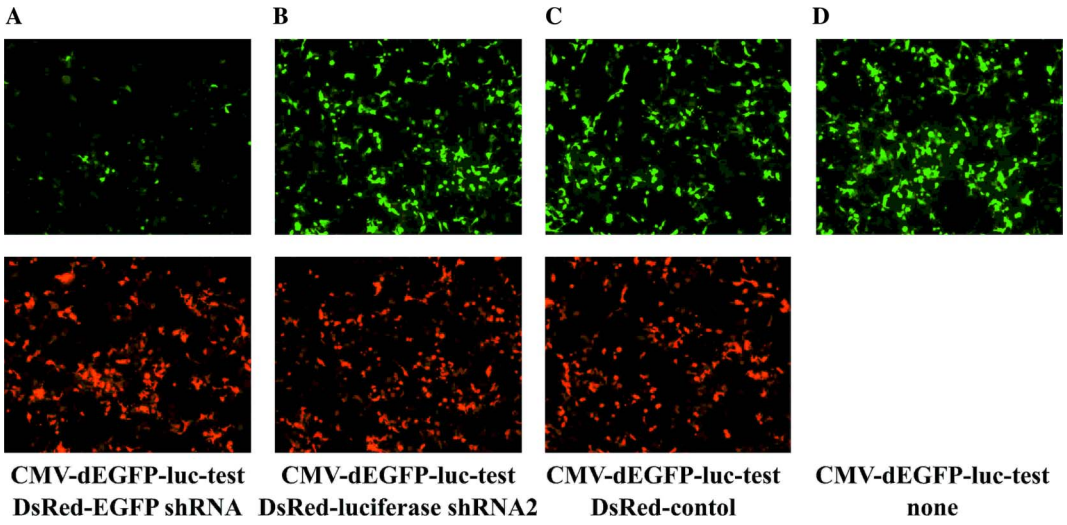

Fig. 2. Regulation of destabilised EGFP (dEGFP) fluorescence in HEK cells expressing CMV-directed EGFP-luciferase test transcripts and reporter- or target sequence-specific shRNAs. HEK 293 cells were transfected with a plasmid expressing the reporter dEGFP and the long (472 bp) luciferase target sequence together with a plasmid expressing an shRNA against the reporter sequence EGFP (lane A), or an shRNA directed against the long target sequence of luciferase (lane B), or a DsRed expressing control plasmid (lane C), or without a second plasmid (lane D). The upper lane shows the CMV-directed dEGFP expression two days after transfection of the cells; the lower lane shows the human synapsin promoter-directed DsRed expression of the shRNA vectors (lanes A and B), or the control vector (lane C), respectively.

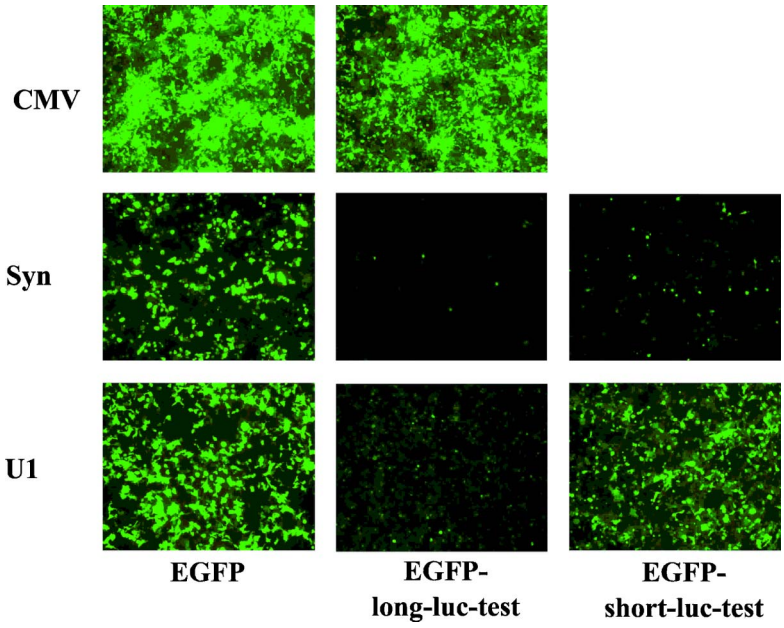

Fig. 3. EGFP expression as a function of promoter and size of the target sequence. HEK cells were transfected with vectors expressing EGFP under the control of the mouse CMV promoter (upper lane, CMV), the human synapsin promoter (middle lane, hSyn), or the human U1 promoter (lower lane, hU1). EGFP fluorescence was analysed two days after transfection of the cells in the absence of a target sequence (lane A, EGFP), in the presence of a long (lane B, target sequence with 472 bp) or a short luciferase target sequence (lane C, target sequence with 121 bp).

When cells were transfected with an EGFP-short-RhoA-expressing test vector (pGEM-U1-EGFP-short-RhoA-test1) together with one of three different RhoA shRNA-expressing vectors, none of the RhoA shRNAs had any obvious effect on the expression level of EGFP, although the shRNA directed against EGFP caused an almost complete down-regulation of EGFP expression (Fig. 5). This result was surprising because all three shRNA sequences were selected using publicly available design tools, and one shRNA even corresponded to a sequence that was already shown to cause an obvious phenotype in vivo and in vitro [22] in a different species. We therefore presumed that either shortening of the target sequence or the combination of promoter, reporter sequence, and target sequence, or both together, prevented an efficient regulation of the test transcript.

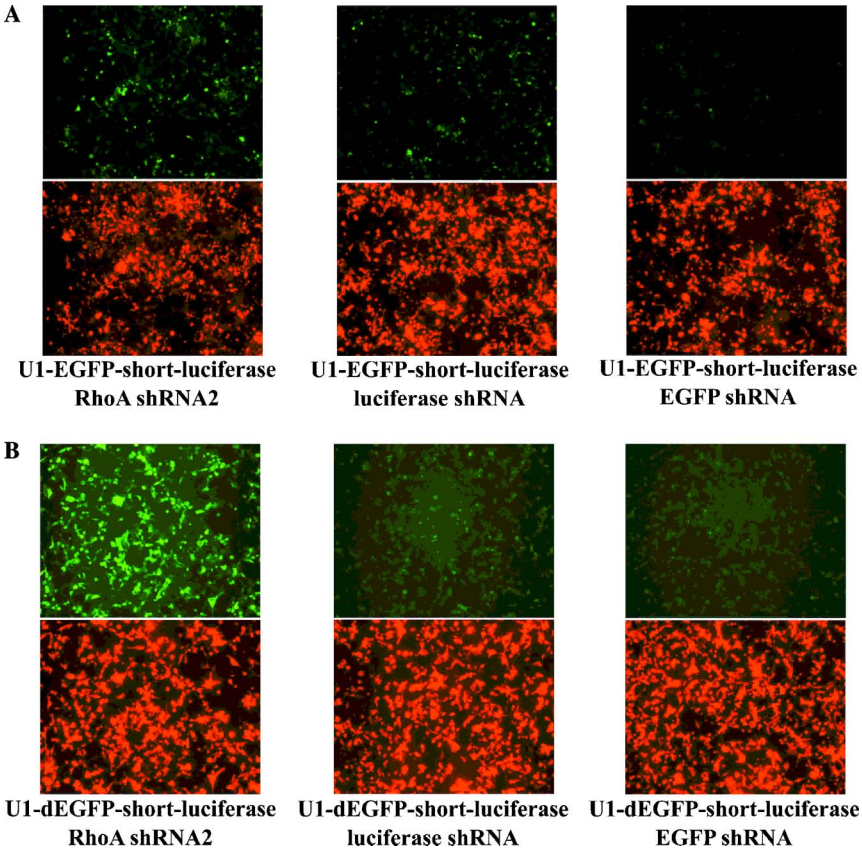

Fig. 4. Regulation of EGFP fluorescence by target sequence- and reporter-specific shRNAs in HEK cells expressing human U1 promoter-directed EGFP-short luciferase test transcripts. Cells were transfected with a plasmid expressing either the reporter EGFP (A) or destabilised EGFP (B) and a short luciferase target sequence (121 bp), together with a plasmid expressing a control shRNA (left lane, RhoA2 shRNA), or a target sequence-specific shRNA (middle lane, luciferase shRNA2), or a reporter sequence-specific shRNA (right lane, EGFP shRNA). The upper lanes of (A,B) depict the human U1 promoter-directed EGFP (A) or dEGFP (B) expression, respectively, the lower lanes depict the human synapsin promoter-directed DsRed expression by the shRNA plasmids two days after transfection of the cells. Please note that the exposure time for the images with dEGFP fluorescence (upper lane in B) was three times longer than for those showing EGFP fluorescence (upper lane in A).

As shown above, test systems expressing EGFP or dEGFP as read-out have obvious limitations, and not every combination of promoter and EGFP target sequences was useful for the analysis of shRNAs. We therefore constructed test vectors containing firefly luciferase instead of EGFP as a reporter and EGFP as a target sequence. The transcription of the luciferase-EGFP transcripts by these vectors was directed by one of the following three promoters: SV40, U1, or CMV (pGL3-SV40-luc-EGFP-test, pGL3-U1-luc-EGFP-test, and pGL3-CMV-luc-EGFP-test). When the basal luciferase expression of these vectors in HEK cells was analysed, the ratios of expression of pGL3-SV40-luc-EGFP-test to pGL3-U1-luc-EGFP-test were 1:7 on day one and 1:10 on days two and three, whereas the ratios of luciferase expression by pGL3-SV40-luc-EGFP-test compared to pGL3-CMV-luc-EGFP-test were already about 1:650 on day 1, 1:750 on day two and 1:850 on day three. Next, we examined the level of luciferase expression using the different vectors in the presence of shRNAs that were directed against either the EGFP target sequence (pAAV-hSyn-DsRed-shRNA-EGFP) or the luciferase reporter sequence (pAAV-hSyn-DSRed-shRNA-luc2), or that had no target sequence (pAAV-hSyn-DsRed-shRNA-RhoA2 as negative control). As shown in Table 2, the difference in luciferase expression of the SV40 promoter- and U1 promoter constructs seen in the presence or absence of the reporter-specific luciferase shRNA increased over a period of three days, whereas it decreased with the CMV promoter-directed target sequence. Furthermore, when the SV40 promoter construct or the U1 promoter construct was used, the range of regulation with the reporter-specific shRNA was between 85% and 90% and between 90% and 95%, respectively, while when the CMV promoter construct was used, the range of regulation was only between 50% and 70% with the reporter-specific shRNA (Table2). The range of regulation with the target sequence-specific EGFP shRNA was between 70% and 80% with the U1 and SV40 constructs, whereas it was hardly above 50% with the CMV promoter construct. Therefore, we used only U1 promoter or SV40 promoter constructs for all further experiments.

The results shown in Fig. 5 prompted us to analyse the knock-down capacity of the three different RhoA-specific

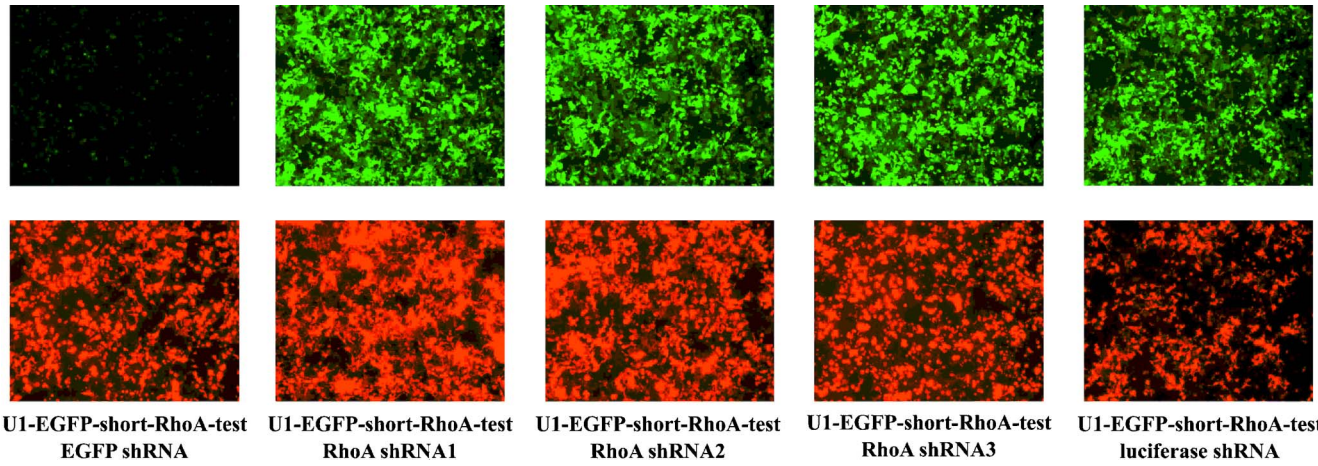

Fig. 5. Regulation of EGFP fluorescence by different RhoA-specific shRNAs in HEK cells expressing U1 promoter-directed EGFP-short RhoA test transcripts. Cells were transfected with a plasmid expressing EGFP as a reporter and a short RhoA-specific target sequence together with a reporter-specific shRNA vector (left lane, EGFP shRNA), or with one out of three different RhoA-specific shRNA vectors (second left lane, RhoA shRNA1; middle lane, RhoA shRNA2; and second right lane RhoA shRNA3), or a control shRNA vector (right lane, luciferase shRNA). The upper lane depicts the human U1 promoter-directed EGFP expression, the lower lane depicts the human synapsin-directed DsRed expression by the shRNA plasmids two days after transfection of the cells.

Table 2
Regulation of SV40-, U1-, and CMV-directed luciferase expression by target- and reporter-specific shRNAs

	SV40 promoter			U1 promoter			CMV promoter		
	day1	day2	day3	day1	day2	day3	day1	day2	day3
Luc shRNA	15 ± 1	9 ± 1	10 ± 1	11 ± 1	6 ± 1	7	30	41	52 ± 4
EGFP shRNA	28 ± 3	20 ± 1	22 ± 1	25 ± 1	21 ± 0	22 ± 1	46 ± 1	47 ± 2	54 ± 9
RhoA shRNA	100 ± 16	100 ± 10	100 ± 4	100 ± 10	100 ± 6	100 ± 4	100 ± 1	100 ± 5	100 ± 5

Influence of the type of promoter on the regulatory range of the luciferase reporter by reporter- and target sequence-specific shRNAs. Cells were transfected with pGL3-SV40-luc-EGFP-test (column 1), pGL3-U1-luc-EGFP-test (column 2), or pGL3-CMV-luc-EGFP-test (column 3) together with one out of three different shRNA-expressing vectors. ShRNA vectors expressed either the reporter-specific luciferase shRNA2 (third lane), the target sequence-specific EGFP shRNA (fourth lane), or the unspecific control RhoA shRNA2. Luciferase activity was measured 24 h (first sub-column), 48 h (second sub-column), or 72 h (third sub-column) after transfection. The luciferase activity measured with the control shRNA RhoA2 was set at 100%. Numbers represent means ± SD from three independent measurements; when no number is given for the SD, the value was below 1%.

shRNAs with the luciferase test system. We cloned vectors that expressed SV40 promoter-directed transcripts of luciferase as a reporter fused to either a short (164 bp) or a long (582 bp, which corresponds to the complete ORF) target sequence of RhoA (pGL3-SV40-luc-long-RhoA-test and pGL3-SV40-luc-short-RhoA-test1). As shown in Fig. 6, all three different shRNAs against RhoA significantly down-regulated the expression of luciferase, independent of the length of the target sequence. In both test systems, shRNA RhoA3 had the highest knock-down capacity. However, when the long RhoA target sequence was used, both shRNA RhoA1 and RhoA2 were less efficient than shRNA RhoA3, whereas in case of the short RhoA target sequence, shRNA 2 was almost as efficient as shRNA RhoA3, but shRNA1 was less efficient than shRNA RhoA 2 and RhoA3.

In a further set of experiments, we analysed the effect of the length of the target transcript on the expression level and range of regulation of luciferase expression. Similar to the results presented in Fig. 3, Fig. 7 shows that addition of the 582-bp long RhoA-specific target sequence to the luciferase reporter sequence resulted in a significant decrease in luciferase expression by 30–50% as compared to the 164 bp short RhoA target sequence, and hence also in a diminished range of regulation. Despite this reduction, the percent down-regulation of luciferase expression by the reporter-specific luciferase shRNA was almost identical in both test systems, indicating only a marginal influence of the length of the target sequence on the percentage regulation of the reporter expression by the reporter-specific shRNA. In contrast, percentage regulation by the shRNA directed against the long and short RhoA target sequence was significantly different in both test systems and was higher in the test system expressing the short RhoA target sequence. The influence of the primary structure of the target sequence is shown in Fig. 8. Here, the short RhoA target sequence was inverted (antisense orientation of the short RhoA sequence in pGL3-SV40-luc-short-RhoA-test2) as compared to the test vector in the experiments shown in Figs. 6 and 7. The results revealed that the range of regulation by luciferase shRNA was not dependent on the primary structure of the target sequence, but the pat-

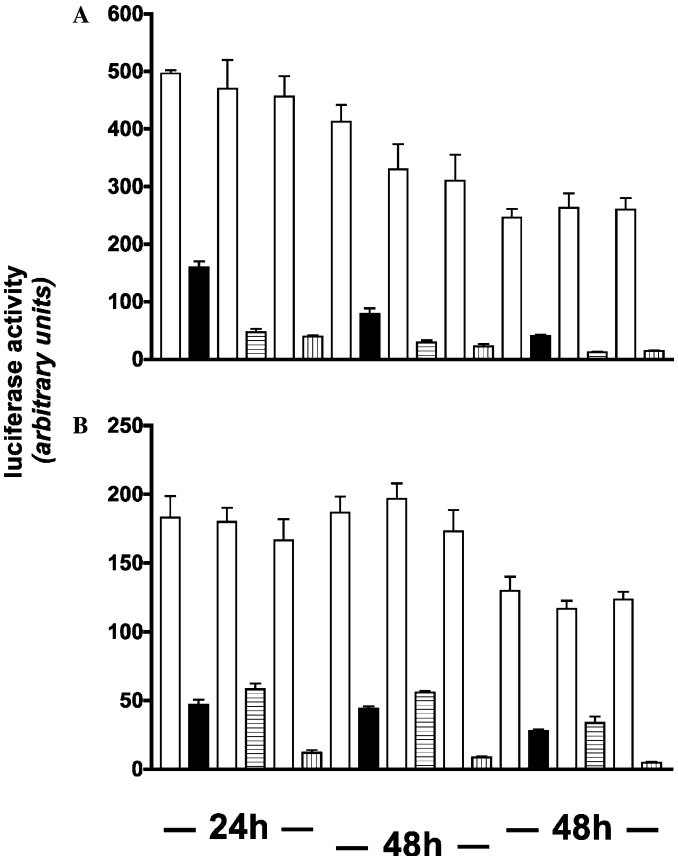

Fig. 6. Regulation of luciferase expression by RhoA-specific siRNAs in HEK cells expressing SV40 promoter-directed luciferase-RhoA-test sequences. Cells were transfected with a plasmid expressing luciferase firefly as a reporter and either a short (A, 162 bp) or a long (B, 582 bp; complete ORF) RhoA-specific target sequence together with a plasmid expressing a control shRNA (shRNA EGFP, white columns), or one out of three different RhoA-specific shRNAs (shRNA RhoA1, black columns; shRNA RhoA2, horizontally striped; shRNA RhoA3, vertically striped). Luciferase expression is shown on the *Y* axis in arbitrary units. Measurements were done 24, 48, and 72 h after transfection of the cells.

tern of regulation by the three different shRNAs against RhoA clearly differed from the pattern observed with the sense-orientated target sequence (Fig. 6A).

Discussion

An RNAi test system should fulfil several criteria in order to facilitate the screening of siRNAs/shRNAs targeted towards a gene of interest. First, it should be possible to carry out the analysis within a reasonable time. Second, the reporter protein should be either directly visible (use of fluorescent proteins) or easy to measure (as for example, with enzyme assays). Third, the cloning of target sequences should be versatile and straightforward, and should avoid translational fusions, in which additional sequences might influence reporter expression ([23] and unpublished observations). Fourth, the dynamic range of regulation of the test system, that is, the difference in expression of the reporter in the presence or absence of a target sequence-specific shRNA, should be large enough to discriminate

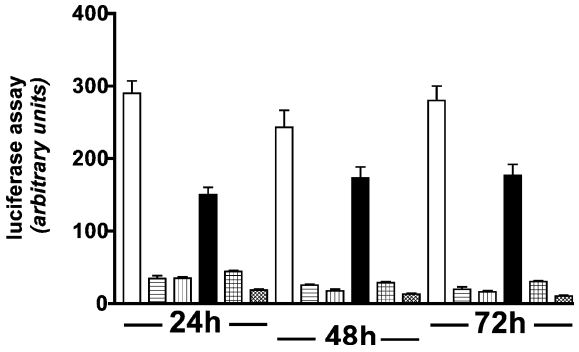

Fig. 7. Influence of the length of the target sequence on the range of regulation of luciferase expression by reporter- and target sequence-specific shRNAs. The white columns represent the luciferase level of cells transfected with pGL3-SV40-luc-short-RhoA-test1 plus pAAV-hSyn-DsRed-shRNA-EGFP, the horizontally striped columns represent the luciferase level of cells transfected with pGL3-SV40-luc-short-RhoA-test1 plus pAAV-hSyn-DsRed-shRNA-RhoA2, and the vertically striped bars represent the luciferase level of cells transfected with pGL3-SV40-luc-short-RhoA-test1 plus pAAV-hSyn-DsRed-shRNA-luc2. The black columns represent the luciferase level of cells transfected with pGL3-SV40-luc-long-RhoA-test plus pAAV-hSyn-DsRed-shRNA-EGFP, the chequered columns represent the luciferase level of cells transfected with pGL3-SV40-luc-long-RhoA-test plus pAAV-hSyn-DsRed-shRNA-RhoA2, and the double-hatched columns represent the luciferase level of cells transfected with pGL3-SV40-luc-long-RhoA-test plus pAAV-hSyn-DsRed-shRNA-luc2. Luciferase expression is shown on the Y-axis in arbitrary units. Measurements were done 24, 48, and 72 h after transfection of cells. For a more detailed description of the plasmids please, see Table 1.

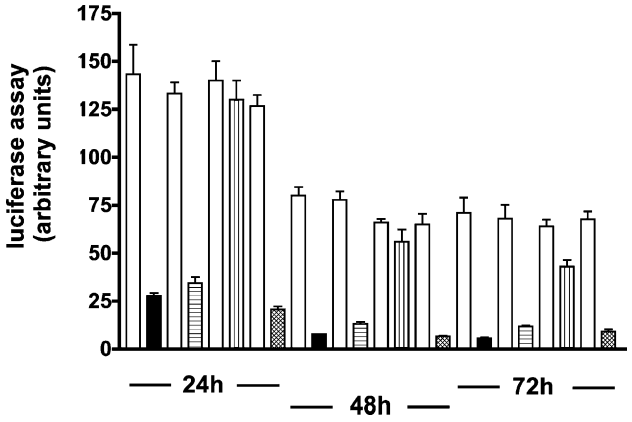

Fig. 8. Influence of the primary structure of the target sequence on the regulation of reporter expression by target sequence-specific shRNAs. Cells were transfected with a plasmid expressing luciferase firefly as a reporter and the inverted form of the short RhoA-specific target sequence together with a plasmid expressing a control shRNA (shRNA EGFP, white columns), or one out of three different RhoA-specific shRNAs (shRNA RhoA1, black columns; shRNA RhoA2, horizontally striped; and shRNA RhoA3, vertically striped), or the reporter-specific shRNA (shRNA luciferase, double-hatched). Luciferase expression is shown on the *Y* axis in arbitrary units. Measurements were done 24, 48, and 72 h after transfection of the cells.

between siRNAs with different knock-down capacities. Fifth, it should be possible to clone long-enough target sequences to account for possible secondary structures in a target RNA that might affect siRNA binding [3,24].

Sixth, the transient transfection of the reporter vector (containing the test system) together with an shRNA-expressing vector or an siRNA, respectively, should be efficient, easy to perform, and cost-effective. Seventh, off-target effects should be avoided and, therefore, cells lacking an interferon response, such as the HEK cells used in this study, should be preferred. Although several RNAi test systems have recently been developed [11,25,26], or have become commercially available, many of them do not fulfil the criteria mentioned above. Furthermore, as we showed here, some are even unsuitable for testing RNAi effects.

Our experiments with the CMV promoter-EGFP reporter test systems revealed that the combination of promoter and reporter is most critical for successful testing of siRNAs/shRNAs. Strong promoters appear to be needed to generate sufficient EGFP expression [23]. Since, on the other hand, knock-down of highly expressed and stable reporter proteins is difficult to achieve, test systems based on CMV-directed EGFP/DsRed expression may not be suitable for testing shRNAs/siRNAs. Even substitution of the stable EGFP by dEGFP with a half-life period of only two hours does not solve the problems of CMV promoter-based test systems, and only shRNAs targeted directly against the reporter sequence of dEGFP can lead to an obvious down-regulation of the EGFP fluorescence. RNAi cleavage products can be detected in cells [3], for which reason intact open-reading frames of targeted transcripts might still be translated at levels high enough to maintain a phenotype, despite RNA cleavage.

Test systems based on EGFP/DsRed fluorescence and promoters weaker than the CMV promoter, such as human U1, simian virus40, or the human synapsin promoter, are of restricted use for shRNA testing. As shown here, the expression of fusion transcripts with longer target sequences is critical, because the expression of the reporter can drop to low or even undetectable levels. Very small target sequences, as they have recently been used, partially circumvent the problem [26] but do not account for the specific properties of the transcript [3,24] and, therefore, are not recommendable. Both, the occasional occurrence of low transfection levels and the difficulty of grading the potency of shRNAs with similar knock-down efficiency, clearly limit the usefulness of fluorescence-based shRNA test systems. The question of why an obvious regulation of the EGFP-short-RhoA transcripts by the three different RhoA-specific siRNAs was not possible, whereas there was evidence of a graded regulation of luciferase-short-RhoA transcripts, remains a matter of speculation. One explanation could be a folding or a too high stability of the target transcript, or both. This result again underlines the importance of an appropriate selection of promoter, reporter, and target sequences, for a useful shRNA/siRNA test system.

The very high level of luciferase expression and the reduced range of regulation seen with CMV-luciferase-based test systems (in comparison to SV40-luciferase and U1-luciferase test systems) point to additional limitations of these test systems. But as shown in the experiments of Table 2, CMV-luciferase test systems are, in contrast to CMV-EGFP test systems, applicable for testing shRNAs, despite the reduced range of regulation and the high level of reporter expression. Nevertheless, because of their larger range of regulation, SV40- and U1-luciferase test systems are preferable.

As in the case of U1 and human synapsin promoter-directed EGFP expression, the expression of luciferase dropped when long target sequences were used instead of short ones. However, whereas in U1-EGFP-based test systems the substitution of a short target sequence by a long target sequence resulted in an almost complete down-regulation of the EGFP fluorescence (Fig. 3), the reduction in luciferase expression caused by the longer target sequences was only 30–50% less than the level measured with short target sequences. The reduction in the absolute amount of expressed luciferase by the long target sequence is concomitant with a reduction in the range of regulation (Fig. 7). In contrast to the length, the primary structure of the target sequence had no obvious influence on the expression of the reporter or the regulation by the reporter-specific shRNA. But the primary structure of the target sequence is, of course, crucial for the regulation of the reporter by the target sequence-specific shRNAs. This is obvious from the experiment shown in Fig. 8 compared to Fig. 6. A comparison of both figures shows that transcripts with the same reporter but sense-and antisense-orientated target sequences are differentially regulated by the same target sequence-specific shRNAs.

In summary, our study shows limitations and possibilities of several shRNA/siRNA test systems, and it clearly demonstrates that some combinations of promoter, reporter, and lengths of target sequences are not useful for the analysis of RNAi effects. Although the EGFP test system has several advantages as compared to the luciferase test system, such as direct detection of fluorescence and the possibility of measuring several time points with the same set of cells, it also has obvious limitations, among them the difficulty of achieving a graded quantification of the RNAi effect. One disadvantage of the luciferase test system is the indirect read-out, which causes extra costs and work. In addition, the luciferase test system needs repeated measurements when time courses are studied. However, one obvious advantage of the luciferase test system is that it allows a more finely graded quantification of shRNAs.

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